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Protocol

Identification of Molecular Markers in Soybean Comparing RFLP, RAPD and AFLP DNA Mapping Techniques

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Abstract. Three different DNA mapping techniques—RFLP, RAPD and AFLP—were used on identical soybean germplasm to compare their ability to identify markers in the development of a genetic linkage map. Polymorphisms present in fourteen different soybean cultivars were demonstrated using all three techniques. AFLP, a novel PCR-based technique, was able to identify multiple polymorphic bands in a denaturing gel using 60 of 64 primer pairs tested. AFLP relies on primers designed in part on sequences for endonuclease restriction sites and on three selective nucleotides. The 60 diagnostic primer pairs tested for AFLP analysis each distinguished on average six polymorphic bands. Using specific primers designed for soybean from *Eco* RI and *Mse* I restriction site sequences and three selective nucleotides, as many as 12 polymorphic bands per primer could be obtained with AFLP techniques. Only 35 % of the RAPD reactions identified

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Abbreviations: AFLP, amplified-fragment-length polymorphisms; PCR, polymerase chain reaction; RAPD, random-amplified-polymorphic DNA; RFLP, restriction-fragment-length polymorphisms; TBE, tris-borate.

a polymorphic band using the same soybean cultivars, and in those positive reactions, typically only one or two polymorphic bands per gel were found. Identification of polymorphic bands using RFLP techniques was the most cumbersome, because Southern blotting and probe hybridization were required. Over 50 % of the soybean RFLP probes examined failed to distinguish even a single polymorphic band, and the RFLP probes that did distinguish polymorphic bands seldom identified more than one polymorphic band. We conclude that, among the three techniques tested, AFLP is the most useful.

The production of genetic linkage maps in plants is a time-consuming but important objective towards the ultimate goal of manipulating gene expression in agronomic crops. The process of establishing a linkage map often entails a combination of placing cDNA sequences of known function, anonymous cDNAs, phenotypic traits, isozymes, and other markers to find linkages among important chromosomal traits.

Previously, restriction-fragment-length polymorphisms (RFLPs) have been routinely used for mapping genomes of many agronomic crops, including tomato (Tanksley et al., 1992), corn (Ahn and Tanksley, 1993), rice (Ahn and Tanksley, 1993), and soybean (Lark et al., 1993; Shoemaker and Olson, 1993; Shoemaker and Specht, 1995). RFLP mapping is often used to place cloned cDNAs encoding proteins of known and unknown function on genetic linkage maps. RFLP techniques have, however, the disadvantage of requiring a great deal of operator time and effort without producing a large number of polymorphisms that can be used as linkage traits.

Another technique developed to generate markers used to establish genetic linkage maps uses PCR to produce random-amplified-polymorphic DNA (RAPD) markers (Williams et al., 1990). RAPD markers are generated using short DNA primers and the polymerase chain reaction (PCR). These markers are easily generated, can be rapidly analyzed, and use only small amounts of DNA. Reproducibility between laboratories, however, can be a problem.

Recently a PCR-based technique of generating molecular markers, amplified-fragment-length polymorphisms (AFLP), was reported (Vos et al., 1995). This powerful DNA fingerprinting technique has been used with plant and bacterial DNAs (Vos et al., 1995; Lin and Kuo, 1995). The AFLP technique is composed of three major steps:

- Restriction endonuclease digestion of genomic DNA followed by ligation of adapters;

- PCR amplification of the restriction fragments; and
- Analysis of the amplified fragments on polyacrylamide gels.

By the combination of different restriction enzymes and the numbers of selective nucleotides in the primers for PCR amplification, DNA analysis by AFLP offers the possibility of a more efficient system for generating molecular markers.

In this article we compare the application of AFLP, RFLP, and RAPD techniques to DNA mapping in soybean (*Glycine max* L. Merr.), an agronomically important crop plant with limited genetic diversity in the cultivars popularly grown in the US. The limited diversity of US soybean germplasm has made the identification of genetic markers more difficult for this crop, and make it a prime candidate to use as a model system in establishing linked traits.

Experimental Procedures

Solution required

TBE: 0.089 tris-borate, 0.025 M EDTA, pH 8.3

Plant material

Fourteen cultivars of soybean (*Glycine max* L. Merr.) were grown in the greenhouse under a 14-hour day length, and the leaf tissue was harvested for genomic DNA isolation. The cultivars analyzed using AFLP, RFLP and RAPD techniques were BARC 5, BARC 4b, Williams, Williams 79, Williams 82, PI 90763, PI 83495, PI 88788, T135, Bass, Essex, Holladay, Hytest 4359, and Morgan, and were obtained courtesy of Thomas Devine (USDA, Beltsville, MD, USA). Two of the soybean cultivars, PI29136 (Noir) and BARC-2 (*Rj4*) (Weisemann et al. , 1992) were crossed, and 13 members representing F_2 and F_3 progeny, along with the parental cultivars, were subjected to analysis by AFLP, RFLP, and RAPD .

Isolation of genomic DNA

Genomic DNA from soybean leaves was isolated using CTAB (hexadecyltrimethylammonium bromide) as previously described (Weisemann et al., 1992).

Polymorphism analysis

RFLP analysis. Restriction endonuclease digestion of genomic DNA with Hae *III* was conducted according to manufacturer's instructions

(Life Technologies, Inc., Gaithersburg, MD, USA). Digests were incubated for 3 hr using 2 to 5 units of enzyme per µg DNA. DNA fragments were separated according to size by electrophoresis in a 1 % agarose gel using TBE running buffer (90 mM tris, 90 mM borate, 2 mM EDTA) for approximately 4 hr at 90 mA (150 V). The DNA was transferred to Tropix Propilon Plus (Tropix, Bedford, MA, USA) nylon membrane, and analyzed using chemiluminescence as described by Dubitsky et al. (1992). RFLP probe pBLT62, a 2400-bp random genomic clone, was labeled using digoxigenin at a ratio of dUTP-Dig:dTTP of 1:9 (10 µM:90 µM) using alkali-labile dUTP-Dig from Boehinger Mannheim (Indianapolis, IN, USA) (Genius Handbook). Probe hybridization was detected by chemiluminescence according to manufacturer's instructions (Boehringer Mannheim, Germany).

RAPD analysis: PCR reactions for RAPD analysis were performed after the method of Williams et al. (1990). PCR reactions of 25 µL contained 15 mM tris HCl, pH 8.3, 6 mM KCl, 2.75 mM $MgCl_2$ 6 H_2O, 100 µM dNTPs, 0.8 µM of arbitrary decamers, 80 ng template DNA, 1.8 units of Taq polymerase (Life Technologies, Gaithersburg, MD, USA). Reactions were heated at 95 C for 5 min, and amplified by 45 cycles at:

- 94 C for 1.2 min,
- 34 C for 1.2 min, and
- 72 C for 2.3 min.

Final reactions were incubated at 72 C for 10 min. An aliquot (25 µL) of the reaction was loaded into a 9 % polyacrylamide gel with a running buffer of TBE. Amplified DNA fragments were separated by electrophoresis at 225 V (9.7 V/cm) for 1600 V·hr and silver stained (Heukeshoven and Dernick, 1985). The RAPD primer DNA sequences used were RP151 (GCT GTA GTG T) and RP389 (CGC CCG CAG T).

AFLP analysis: AFLPs were generated using AFLP Analysis System I (Life Technologies, Inc.) following manufacturer's instructions and as described by Lin and Kuo (1995). Soybean genomic DNA (250 ng) was digested by *Eco* RI and *Mse* I at 37 °C for 2 hr, then heated to 70 °C for 15 min to inactivate the enzymes. The DNA fragments were ligated to *Eco* RI and *Mse* I adapters provided in the kit. The ligation mixture was diluted 10-fold with sterile distilled water and the fragments were preamplified by 20 PCR cycles (94 C for 30 s, 56 C for 60 s, 72 C for 60 s) using *Eco* RI+A (5'-GACTGCGTACCAATTC+A 3') and *Mse* I+C (5'-GATGAGTCCTGAGTAA+C 3') primers described by Vos et al. (1995) provided in the kit. Selective amplification was conducted by PCR using

aliquots of the preamplified fragments diluted 50-fold, using ^{32}P-labeled *Eco* RI+3 and unlabeled *Mse* I+3 primers and amplified by PCR using one cycle at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 60 s, followed by lowering the annealing temperature each cycle 0.7 °C for twelve cycles. The reaction was amplified for 23 cycles at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 60 s. The samples were analyzed on a denaturing 5 % polyacrylamide DNA sequencing gel containing 7.5 M urea. Autoradiography was performed by exposing Kodak BioMax MR-2 film (Eastman Kodak Co., Rochester, NY, USA) to the dried gel overnight with intensifying screens. *Mse* I+3 primers were used because they produced numerous bands, but the display of bands was not too dense for reading. *Mse* I+2 primers produced too many bands for good viewing (Lin and Kuo, unpublished data).

Results

Identification of DNA polymorphisms in soybean cultivars

The number of AFLP polymorphic bands produced in a single reaction is determined by three factors:

- The specificity of restriction enzyme primers used to digest the genomic DNA;
- The number and choice of selective nucleotides at the 3' end of the restriction enzyme primers; and
- The size and complexity of the plant genome being analyzed (Vos et al., 1995; Lin and Kuo, 1995).

Of the restriction enzymes available, *Eco* RI and *Mse* I were selected because they provide an optimal number of restriction sites in non-G/C rich regions of genomic DNA from a variety of sources (Vos et al., 1995). These restriction enzymes coupled with selected primers produced multiple polymorphic bands in all of the soybean cultivars used in this study. These restriction enzymes generally yield about 50 to 100 DNA bands on a denaturing sequencing gel when genomic DNA ranging in size from $5x10^8$ to $6x10^9$ bp is used, regardless of the source of the DNA. The size of the soybean genome is about $1.1x10^9$ bp (Arumuganathan and Earle, 1991). When AFLP analysis was performed on a variety of cultivars of soybean, 50 to 100 DNA bands were separated on a sequencing gel when three selective nucleotides were used (Fig.1). More than three selective nucleotides produce fewer bands than that in Fig. 1; thus there would be less chance of displaying different polymorphisms among the cultivars. Using primers with two or one selective nucleotides produces

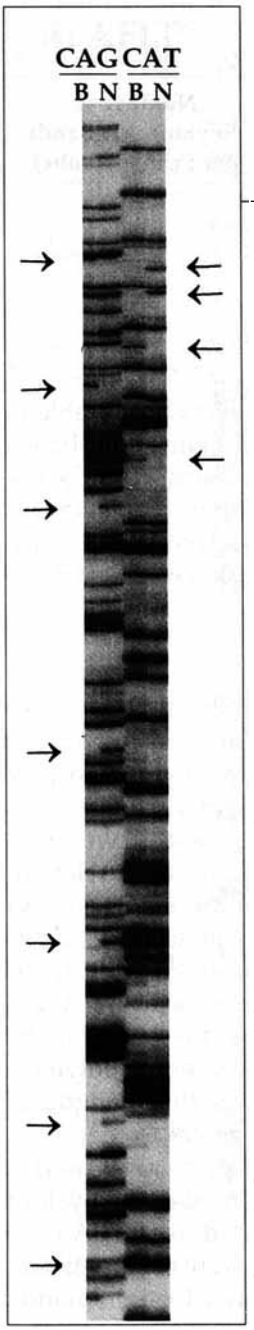


Fig. 1. AFLP polymorphisms of soybean varieties BARC 2 (B) and Noir (N) and using one common selective primer (*Eco* RI+AAC) and a second primer. The AFLP displays are shown using the second primers: CAG, *Mse* I+CAG; CAT, *Mse* I+CAT. Fragments shown are in the 500- to 50-bp range.

excessively high numbers of DNA fragments on gels, thus hindering resolution of the AFLP polymorphic bands.

Abundance of identified polymorphisms

We explored production of molecular markers with AFLPs using several different primer pairs with genomic DNAs of two soybean cultivars, BARC-2 and Noir. These lines were used as parents of a cross made for developing a genetic linkage map of soybean. Multiple polymorphic bands were identified with numerous primer pairs by the analysis of DNA by AFLP (Fig. 1). To evaluate the frequency and abundance of polymorphisms by each technique, genomic DNA from fourteen different soybean cultivars was isolated and subjected to AFLP, RAPD, and RFLP analysis. Ninety-four percent (60 out of 64) of the AFLP primer pairs examined identified polymorphisms (Table I). In most cases these primers yielded multiple polymorphic bands (Fig. 2A). In addition to the results shown, some AFLP primer combinations resulted in as many as 12 polymorphic bands per primer.

RAPD reactions using the same DNA distinguished polymorphisms on the gel from 35 % of the primers tested. When bands were present with the RAPD analysis, there were typically one or two polymorphically different bands displayed per primer (Fig. 2B). Thus the average yield of polymorphisms present per primer examined was 0.7 (Table I). Similar results were obtained using a variety of other RAPD primers.

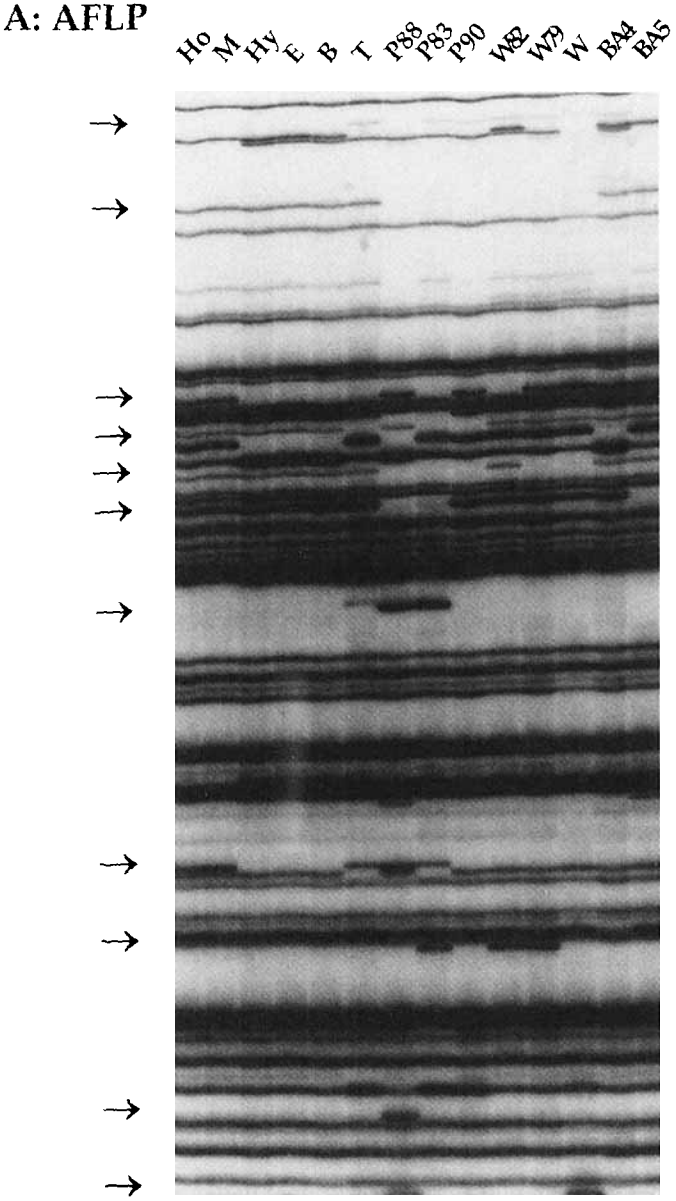
Table I. Comparison of RFLP, RAPD, and AFLP.

DNA Technique	Number of Primers (Probes) Examined	With Polymorphic Bands	Percent of Total	Number of Polymorphic Bands per Primer (Probe)
RFLP	209	104	50	0.5
RAPD	245	85	35	0.7
AFLP	64	60	94	5.6

When RFLP probes were tested, 50 % of the probes were unable to identify a single polymorphism, and the number of polymorphic bands per positive probe was even less than with RAPD reactions (Fig. 2C). The ratio of polymorphisms that were identified per RFLP probe tested was close to 0.5 (Table I). Thus, the AFLP DNA analysis technique consistently yielded more polymorphic markers than did either RAPD or RFLP procedures.

Inheritance of identified polymorphisms

In addition to differentiating between various cultivars, DNA analysis has been used extensively in the past to follow inheritance patterns between closely related crosses. To construct a useful polymorphic genetic linkage map in soybean that is both efficient and reproducible, the molecular markers used must be present in the parents as well as in offspring in a sufficient number to rapidly identify inheritance relationships. To this end it is necessary to demonstrate the inheritance of molecular markers in offspring as well as parents. Therefore, genomic DNAs isolated from F_2 and F_3 plants of a cross between PI290136 (Noir I) X BARC-2(*Rj4*) (Weisemann et al., 1992) were examined by AFLP, RAPD, and RFLP techniques (Fig.3). AFLP primers *Eco* RI+ACC and *Mse* I+CAC (different from that used in Fig.2) were used to identify polymorphic bands in parents and offspring (Fig. 3A). One of the strengths of AFLP analysis of DNA is the ability to change primers to identify a different set of polymorphic bands. The *Eco* RI+ACC primer was used in Figs. 2 and 3, while the second primer (*Mse* I+CTT) used for analysis of soybean cultivars in Fig. 2 is different from that used in Fig. 3A (*Mse* I+CAC). With *Mse* I+CAC four polymorphic bands were differentiated. These bands segregated and could be readily scored for inheritance.


A: AFLP
Ho
M
Hy
E
B
T
P88
P83
P90
W2
W9
W
BA4
BA5

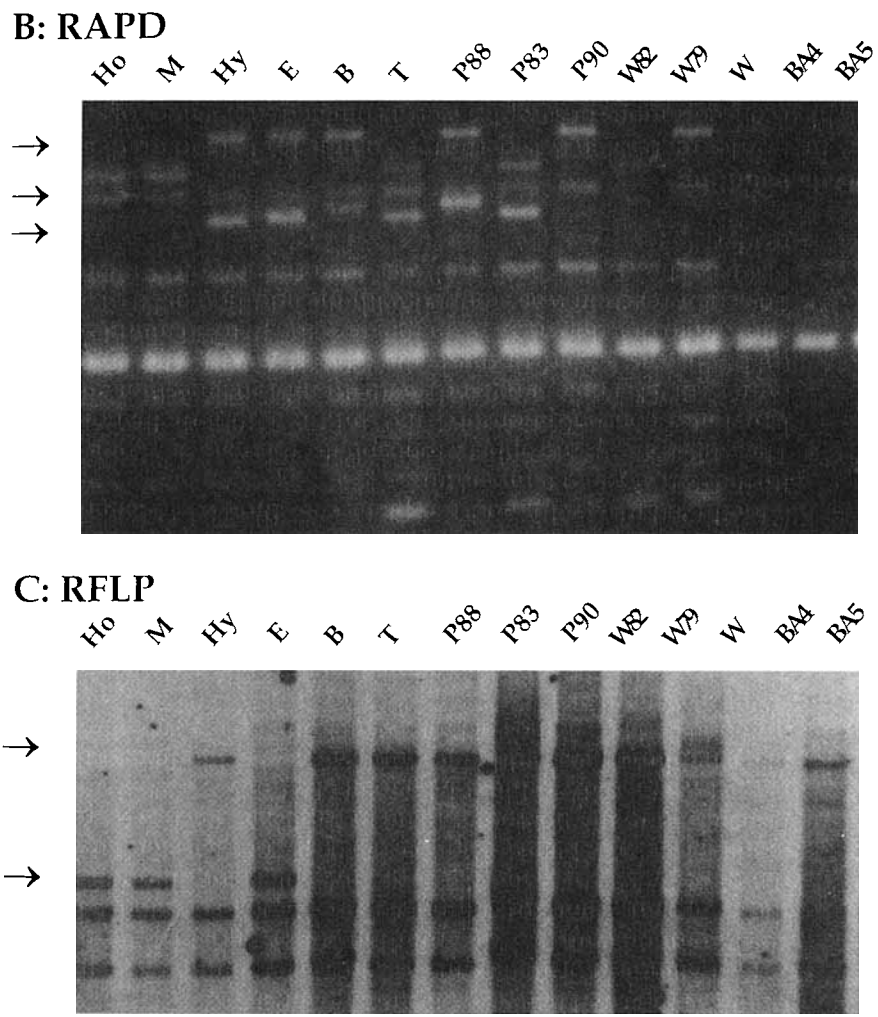
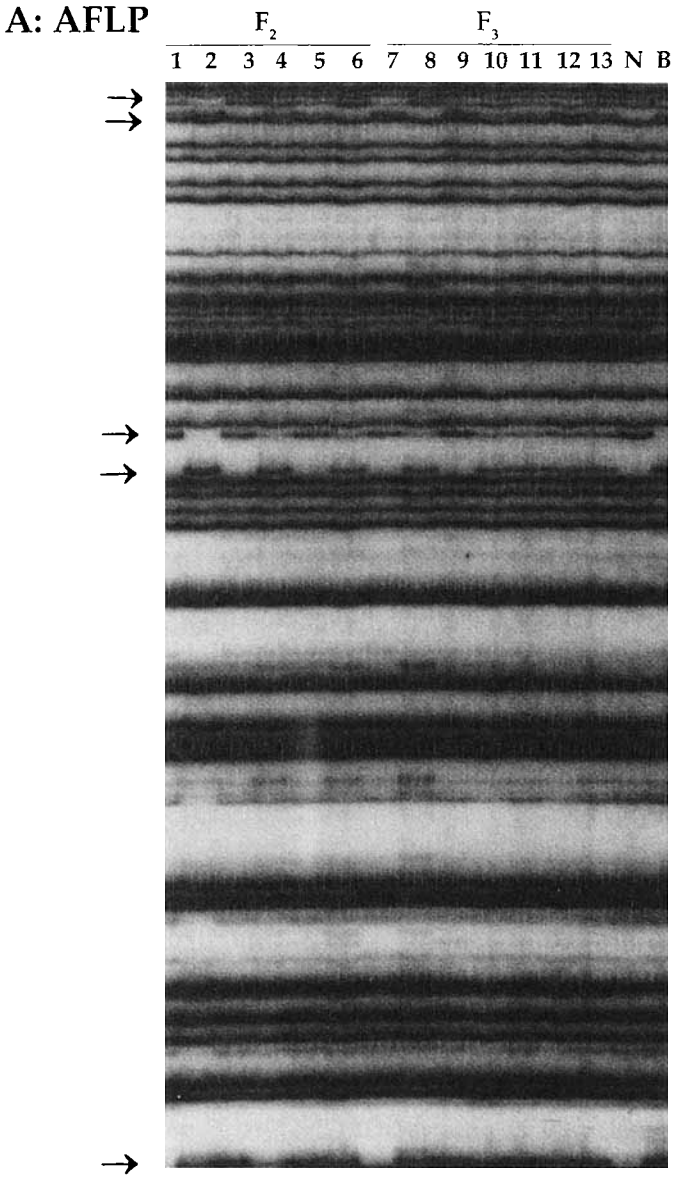


Fig. 2. Polymorphisms found in different soybean cultivars using AFLP, RAPD, and RFLP techniques. Ho, Holladay; M, Morgan; Hy, Hytest; E, Essex; B, Bass; T, T135; P88, PI 88788; P83, PI 83495; P90, PI 90763; W82, Williams 82, W79, Williams 79; W, Williams; BA4, BARC-4b; BA5, BARC-5. Panel A displays AFLPs; Panel B displays RAPDs; Panel C displays RFLPs. AFLP was performed on 250 ng of genomic DNA and amplified with selective primers of *Eco* RI+ACC and *Mse* I+CTT. RAPD was performed on 80 ng of genomic DNA using the primers GCT GTA GTG T and CGC CCG CAG T. RFLP was performed using 10 µg genomic DNA, digested with *Hae* III and hybridized with digoxigenin-labeled pBLT62. The hybridization pattern was detected by chemiluminescence.


A: AFLP
F2
F3
1 2 3 4 5 6 7 8 9 10 11 12 13 N B

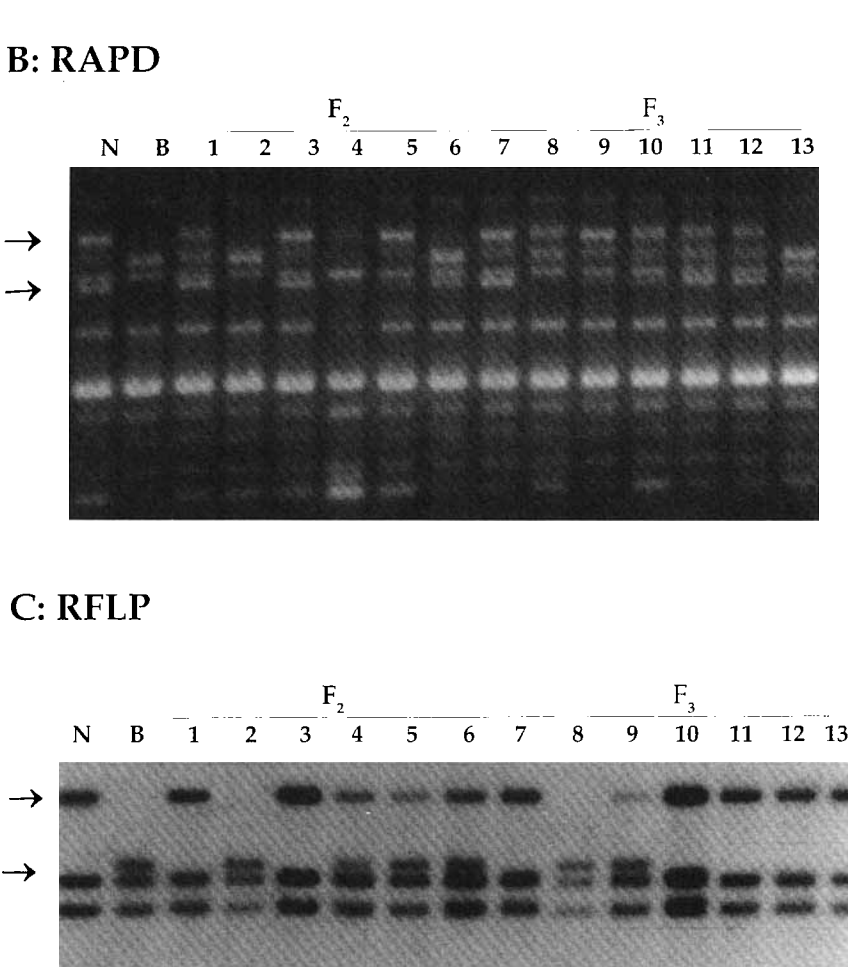


Fig. 3. The polymorphisms of genomic DNA from F_2 and F_3 plants of a segregating cross Noir x BARC-2. Lanes 1-6 represent F_2 plants; lanes 7-13 represent F_3 plants; parental plants are Noir (N) and BARC-2 (B). Panel A: AFLP was performed on 500 ng of genomic DNA and amplified with selective primers *Eco* RI+ACC and *Mse* I+CAC. Panel B: RAPD was performed on 80 ng of genomic DNA and amplified with the primers GCT GTA GTG T and CGC CCG CAG T. Panel C: RFLP was conducted using 10 µg of genomic DNA, digested with *Hae III* and hybridized with digoxigenin-labeled pBLT62. The hybridization pattern was detected by chemiluminescence.

When the same progeny and parents were analyzed by RAPD techniques, two polymorphic markers were seen (Fig. 3B). Similarly, two RFLP markers could be scored (Fig. 3C).

Discussion

Genetic linkage maps and DNA typing to identify closely related cultivars requires an abundance of polymorphic traits that can be obtained in a rapid and reliable fashion. Molecular polymorphic DNA markers have been used effectively for genetic linkage mapping, and AFLP, RAPD, and RFLP techniques are all capable of producing multiple, distinct DNA polymorphisms. Each technique, however, has its own positive and negative features.

Identifying RAPD markers is relatively simple, since it only involves PCR and agarose gel electrophoresis. In addition, RAPD polymorphisms are usually visualized without the need of labelled radioisotopes, which can be a significant handling and disposal concern. Reproducibility of the RAPD markers is, however, dependent upon the annealing of 9- to 10-b arbitrary sequences of the primers. Primers of a higher AT composition anneal at a lower temperature than do those with higher GC composition. It becomes more difficult, therefore, to obtain repeatable results using standard PCR conditions as the number of primers screened increases. In addition, subtle differences in Mg^{++} concentration, dNTP concentration, cycling parameters, and other conditions significantly affect performance of the PCR reaction, affecting the reproducibility of some RAPD analyses and the transfer of RAPD identities among laboratories.

Detection of RFLP markers involves preparation and labelling of DNA probes, digestion of numerous genomic DNAs with restriction enzymes, gel electrophoresis, and Southern blot hybridization. The process is more labor intensive than RAPD DNA analysis and requires large amounts of genomic DNA (5–10 μg per plant). In addition, identifying polymorphic RFLP DNA probes can be expensive and time-consuming. Based on this report and others (Keim et al., 1990; Apuya et al., 1988), the number of RFLP polymorphisms identified per probe is low; it is useful, nonetheless, for placing DNA sequences of known function on genetic linkage maps.

Using the AFLP technique, we have introduced short, defined sequences as adapters onto enzyme-digested genomic DNA. The restric-

tion enzymes selected for the digestion (*Eco* RI and *Mse* I) are particularly stable and well suited for non-GC rich regions of DNA. The desired DNA fragments are selectively amplified by PCR using primers of defined sequences, resulting in highly reproducible DNA fragment patterns using standard PCR conditions. Use of a high-resolution sequencing gel allows large numbers of amplified DNA fragments to be separated for detection. The percentage of reactions yielding useful polymorphisms is very high, and the presence of multiple polymorphic bands per reaction add to the efficiency of the technique.

High reproducibility, rapid generation, and high frequency of identifiable AFLP polymorphisms make AFLP DNA analysis an attractive technique for identifying polymorphisms and for determining linkages by analyzing individuals from a segregating population. AFLP analysis does require technical expertise for pouring sequencing gels and using radioactive label to detect the polymorphisms on an autoradiogram. AFLP markers were readily scored in segregating populations to determine inheritance of the polymorphism. Because multiple polymorphic bands were resolved for each segregating sibling in a single gel lane, multiple loci could be mapped at the same time, increasing the efficiency of determining linkages to form a map. Additional developments of AFLP DNA mapping procedures will enhance the efficiency and ease of the technique, ensuring its wider use in genome mapping programs.

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